THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 34, Issue of August 25, pp. 26082–26088, 2000

Printed in U.S.A.

Disulfide Bonds Are Generated by Quinone Reduction*

Received for publication, May 5, 2000, and in revised form, June 13, 2000
Published, JBC Papers in Press, June 14, 2000, DOI 10.1074/jbc.M003850200

Martin W. Bader‡, Tong Xie§, Chang-An Yu§, and James C. A. Bardwell‡¶

From the ‡Department of Biology, University of Michigan, Ann Arbor, Michigan 48109-1048 and the §Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078

The chemistry of disulfide exchange in biological systems is well studied. However, very little information is available concerning the actual origin of disulfide bonds. Here we show that DsbB, a protein required for disulfide bond formation *in vivo*, uses the oxidizing power of quinones to generate disulfides *de novo*. This is a novel catalytic activity, which to our knowledge has not yet been described. This catalytic activity is apparently the major source of disulfides *in vivo*. We developed a new assay to characterize further this previously undescribed enzymatic activity, and we show that quinones get reduced during the course of the reaction. DsbB contains a single high affinity quinone-binding site. We reconstitute oxidative folding *in vitro* in the presence of the following components that are necessary *in vivo*: DsbA, DsbB, and quinone. We show that the oxidative refolding of ribonuclease A is catalyzed by this system in a quinone-dependent manner. The disulfide isomerase DsbC is required to regain ribonuclease activity suggesting that the DsbA-DsbB system introduces at least some non-native disulfide bonds. We show that the oxidative and isomerase systems are kinetically isolated *in vitro*. This helps explain how the cell avoids oxidative inactivation of the disulfide isomerization pathway.

Much progress has been made in our understanding of how disulfide bonds are formed during protein folding in the cell. In *Escherichia coli*, a number of Dsb proteins catalyze the oxidation, reduction, and isomerization of disulfide bonds in newly exported proteins (for recent reviews see Refs. 1–3). Disulfide bond formation is crucial for the structure and stability of many of the proteins in which they are found. In prokaryotes, disulfides form in the rather oxidizing environment of the *E. coli* periplasm (1). The oxidizing power of the periplasm originates from the DsbA-DsbB system (4–6). DsbA is a small soluble protein, which contains a thioredoxin-fold with a highly unstable disulfide bond. DsbA acts by transferring its active site disulfide bond very rapidly to folding proteins. This leads to the oxidation of the target protein and the reduction of the active site C*XX*C motif of DsbA (7–10). The inner membrane protein DsbB reoxidizes the active site C*XX*C motif of DsbA *in vivo* and *in vitro* (5, 11). A similar system is responsible for forming disulfide bonds in the endoplasmic reticulum of eukaryotes (12). Protein disulfide isomerase (PDI)[1] is responsible for the net oxidation of protein thiols *in vivo*. A second protein termed Ero1p that reoxidizes PDI *in vivo* has been identified (13, 14). The disruption of the *ero1* gene leads to the accumulation of reduced PDI and a severe defect in the oxidation of the endoplasmic reticulum protein carboxypeptidase (12). PDI is also capable of isomerizing non-native disulfide bonds (15).

In *E. coli* the identification of an additional three Dsb proteins, DsbC, DsbD, and DsbG, led to the emergence of a second pathway that is responsible for the isomerization of incorrectly formed disulfide bonds in proteins with multiple disulfide bonds (16–19). This system is particularly important for the heterologous expression of eukaryotic proteins containing multiple disulfide bonds such as urokinase (20).

In vivo, DsbA is mainly oxidized, explaining why it functions as an oxidant, whereas DsbC and DsbG are mostly found in their reduced states. This is a prerequisite for their function, since only a reduced isomerase can attack incorrectly formed disulfides (19, 20). How are DsbG or DsbC kept reduced in the oxidizing environment of the bacterial periplasm and where does the oxidative power for the DsbA-DsbB system originate?

The reduction of DsbC and DsbG depends on the presence of the inner membrane protein DsbD (19, 22). DsbD contains six essential cysteines and seems to connect the disulfide isomerization pathway with the reductive power of the cytosol of the cell (22, 23). It was found that the reduction of DsbC also depends on the presence of cytosolic thioredoxin or thioredoxin reductase (22). It has been suggested that thioredoxin keeps the two cytosolic cysteines reduced which in a series of intramolecular disulfide exchange reactions reduce the C*XX*C motif of DsbD which then reduces DsbG or DsbC (22). The existence of DsbD, however, does not explain why the oxidative DsbA-DsbB system is apparently incapable of oxidizing and thus inactivating the isomerization pathway.

Regarding the oxidative pathway, it could be shown that components of the electron transport chain serve as immediate electron acceptors of DsbB (6, 24). By reconstituting the DsbA-DsbB system with purified components *in vitro,* we were able to show that ubiquinone serves as immediate electron acceptor of DsbB (6). Ubiquinones are then reoxidized by terminal oxidases such as cytochrome *bd* and *bo* oxidase which finally transfer electrons onto oxygen. The observation that quinones are electron acceptors of DsbB demonstrates why disulfide bonds still form under anaerobic conditions (6). Menaquinones whose synthesis is up-regulated upon oxygen depletion (25) are able to reoxidize DsbB *in vitro*. Menaquinones are then reoxidized by anaerobic reductases such as fumarate reductase.

Here, we characterize the reoxidation of DsbB by quinones. We report evidence that ubiquinone gets reduced upon DsbB-

* This work was supported by Grants GM 57039 and GM 30721 from the National Institutes of Health (to J. C. B and C. A. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ PEW Scholar. To whom correspondence should be addressed. Tel.: 734-764-8028; Fax: 734-647-0884; E-mail: jbardwel@umich.edu.

[1] The abbreviations used are: PDI, protein disulfide isomerase; DTT, dithiothreitol; drRNase, denatured, reduced ribonuclease; Q_0C_{10}, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; $Q_0C_{10}Br$, 2,3-Dimethoxy-5-methyl-6-(10-bromo)-decyl-1,4-benzoquinone; HPLC, high performance liquid chromatography.

mediated oxidation of DsbA, and we propose a specific binding site for ubiquinones that we titrated with externally added ubiquinone. We further show that the DsbA-DsbB-quinone system efficiently reoxidizes reduced RNase A, but that RNase activity is only regained when the disulfide isomerase DsbC is present.

EXPERIMENTAL PROCEDURES

Purification of Proteins—DsbA and DsbC were purified essentially as described before (11, 30). DsbB was purified from membranes prepared from the DsbB overexpression strain WM76. The membranes were solubilized in 1% *n*-dodecyl maltoside. The His-tagged DsbB was then bound to a nickel-nitrilotriacetic acid column that had been equilibrated with 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.02% *n*-dodecyl maltoside by passing the solubilized membranes over the column at a flow rate of 0.2 ml/min. The column was washed with the same buffer containing 50 mM imidazole. DsbB was eluted with a linear imidazole gradient ranging from 50 to 300 mM. Fractions containing DsbB were pooled and loaded directly onto a hydroxyapatite column equilibrated with 50 mM sodium phosphate, pH 6.2, 100 mM NaCl, and 0.1% *n*-dodecyl maltoside. DsbB was eluted from the column with a linear gradient in a buffer that contained 300 mM NaCl, 0.1% *n*-dodecyl maltoside, and sodium phosphate ranging in concentration from 50 to 500 mM. Fractions containing purified DsbB were concentrated to 5 mg/ml and dialyzed *versus* 10 mM Hepes, pH 7.5, 50 mM NaCl. The protein was stored at −70 °C without loss of activity for >6 months. The DsbB concentration was determined after reduction of protein-bound quinone with $NaBH_4$ using the extinction coefficient of $\epsilon_{276} = 46.5\ mM^{-1}$.

DsbC and DsbA were reduced by incubation in 10 mM DTT for 20 min on ice. Proteins were purified from DTT by PD-10 (Amersham Pharmacia biotech) gel filtration in 20 mM Hepes, pH 7.5, 0.5 mM EDTA. The thiol content was measured with DTNB as described (26). DsbA and DsbC were >95% reduced and stored at −70 °C until use.

Enzymatic Assay—DsbB activity was followed in 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside at 25 °C. Reoxidation of DsbA was measured with a Hitachi fluorescence spectrophotometer as described before (11). The reduction of ubiquinone analogues was measured photometrically (Beckman). Q_0C_{10} reduction was followed at 275 nm with an extinction coefficient of $12.25\ mM^{-1}$. The reaction was started by the addition of a small volume of DsbB. The concentration of DsbB was between 0.5 and 5 nM and was linear to the initial velocity over this range of enzyme concentration.

For steady state kinetics, initial rates were derived from the linear decrease of either fluorescence of DsbA or ubiquinone. The rates were transformed into nM DsbA/s or nM quinone/s and plotted against the concentration of substrate. The data were fitted to a hyperbola, and V_{max} and K_m values were obtained from the fit.

Identification of Ubiquinone Bound to DsbB—Absorbance of DsbB was recorded from 240 to 390 nm in 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside. Ubiquinones were reduced by adding few grains of solid sodium borohydride to the cuvette and mixing thoroughly. After 5 min incubation at room temperature, the reduced spectrum was recorded from 240 to 390 nm. Employing an absorption coefficient of $\Delta\epsilon_{275} = 12.25\ mM^{-1}$, we calculated the amount of bound ubiquinone.

The quinone species bound to DsbB was identified and quantified by high performance liquid chromatography (HPLC). Methanol (−20 °C) was added to 1.4 ml of DsbB (4.4 mg/ml) to yield a final volume of 10 ml and vortexed immediately. Ubiquinone was extracted with 4 × 15 ml of hexane. The sample was dried by evaporation of the solvent and dissolved in 5 ml of diethyl ether. The sample was dried again, dissolved in 0.5 ml of 95% ethanol, filtered through a 0.2-μm membrane, and loaded onto a Microsorb-MV® reverse phase column (C8, 5 μm). Coenzymes Q1, Q2, Q8, and Q10 were used as standard quinone compounds for HPLC. Coenzyme Q8 was extracted from *E. coli* membranes by following a published procedure (27). Ubiquinones were eluted from the column with a linear gradient ranging from 90 to 100% methanol (v/v). The flow rate was 0.8 ml/min. The ubiquinone species bound to DsbB was identified and quantified based on the retention time and peak area of known standard coenzyme Qs.

Titration of Ubiquinone-binding Site of DsbB with External Quinone—Purified DsbB was washed with 10 volumes of titration buffer containing 50 mM K^+/Na^+ phosphate, pH 7.4, 1.0% sodium cholate prior to quinone titration. This was done because sodium cholate generally improves the signal obtained during quinone titrations over that obtained in the presence of dodecyl maltoside. Indeed no signal could be detected for DsbB in the presence of 0.1% dodecyl maltoside, but a good signal was detected in the sodium cholate detergent. Titration experiments were performed in a total volume of 1 ml at a DsbB concentration of 0.44 mg/ml (22 μM). 2,3-Dimethoxy-5-methyl-6-(10-bromo)-decyl-1,4-benzoquinone ($Q_0C_{10}Br$) was added stepwise in 1-μl volumes from a 5 mM stock solution. After the addition of 1 μl of quinone solution, the sample was incubated for 15 min at room temperature, and the spectra were recorded from 240 to 340 nm. The absorbance change at 280 nm upon addition of $Q_0C_{10}Br$ was plotted against the concentration of quinone added to the cuvette. A titration experiment in the absence of DsbB was performed as a control and showed basically the same change in absorbance for each μl of added quinone.

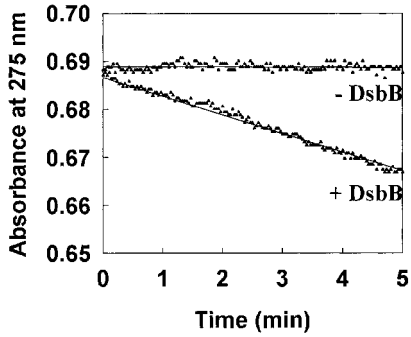

FIG. 1. **DsbB is a novel quinone reductase.** The quinone reductase activity of DsbB was monitored by following the reduction of Q_0C_{10} at 275 nm. Reduced DsbA and Q_0C_{10} were present at 20 μM in 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside at 25 °C. No reaction was observed in the absence of DsbB. After addition of DsbB to a final concentration of 2 nM, reduction of ubiquinone was observed resulting in a linear decline in absorbance at 275 nm. The initial velocity is linear over at least 5 min. From the slope in the presence of DsbB, an activity of 278 nmol of quinone per nmol of DsbB per min was calculated.

Preparation of Denatured and Reduced Ribonuclease A—Ribonuclease A was incubated in 100 mM Tris-HCl, pH 8.0, 6 M guanidine hydrochloride, 120 mM DTT, 0.2 mM EDTA for 1.5 h at 37 °C. The buffer was exchanged to 0.1% acetic acid by PD-10 gel filtration. The column was equilibrated in 0.1% acetic acid. Reduced denatured ribonuclease was quantified by using $\epsilon_{275.5} = 9.3\ mM^{-1}$, whereas an absorbance coefficient of $\epsilon_{275.5} = 9.8\ mM^{-1}$ was used for the native protein. Oxidative refolding was initiated by dilution of drRNase into DsbB assay buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside). The buffer also contains 0.1 μM DsbB, 50 μM Q-1, and 0.1–1.0 μM oxidized DsbA. The final concentration of drRNase was 10 μM. Oxidative refolding was monitored by following the reduction of Q_0C_{10} at 275 nm. Q_0C_{10} reduction was dependent on the presence of catalytic quantities of oxidized DsbA. To test whether ribonuclease gained catalytic activity after exposure to the DsbA-DsbB system, aliquots were taken and diluted 1:10 into the same buffer containing 5 mM cCMP. Native RNase catalyzes the hydrolysis of cCMP that can be monitored at 296 nm ($\Delta\epsilon_{296} = 0.19\ mM^{-1}$). Activity was monitored in the presence or absence of the reduced disulfide isomerase DsbC (10 μM final concentration). Native RNase served as a positive control under the same assay conditions.

RESULTS

Demonstration of a Novel Enzymatic Activity, Ubiquinone-dependent Disulfide Bond Formation—The ultimate source of oxidizing equivalents for the formation of disulfide bonds in the prokaryote *E. coli* originates in the electron transport system (6, 24). By reconstituting the DsbA-DsbB system *in vitro*, we demonstrated that DsbB is reoxidized by quinones (6). Our data suggest that ubiquinones function as the electron acceptors of DsbB under aerobic growth while under anaerobic conditions menaquinones reoxidize DsbB. Enzymatic activity of DsbB can be measured by following the reoxidation of DsbA which results in a decreased relative fluorescence of DsbA upon oxidation (11). Apparently, quinones serve as the second substrate of DsbB, and it can be predicted that quinones should get reduced during the course of the reaction. To demonstrate this, we decided to monitor the reaction by simply following the

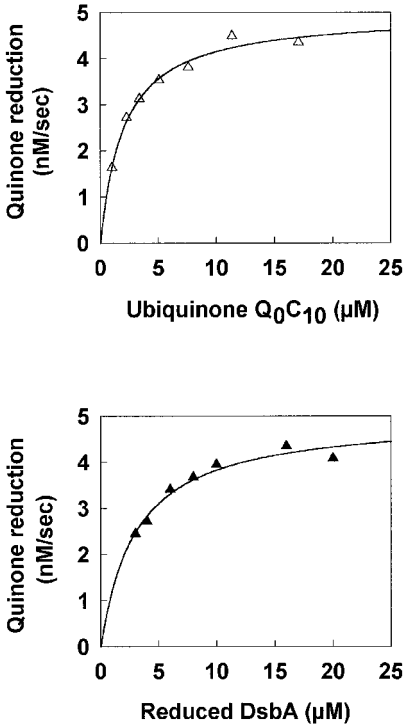

FIG. 2. **Kinetic properties of DsbB determined by the quinone reduction assay.** *A,* initial velocities of DsbB-catalyzed quinone reduction were measured at 275 nm. The assay buffer consists of 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside, and 20 μM reduced DsbA. Q_0C_{10} was added to the indicated concentrations. To start the reaction, DsbB was added to a final concentration of 1 nM. The data were fit to a hyperbola using Sigma Plot (SSPS). The apparent values for $K_m(Q_0C_{10})$ = 2.0 μM and V_{max} = 5.0 nmol of ubiquinone per nmol of DsbB/s were calculated from the fit. *B,* initial velocities of quinone reduction were determined under saturating concentrations of Q_0C_{10} (25 μM). Increasing amounts of reduced DsbA were added, and the reaction was started by the addition of DsbB to 1 nM. The apparent K_m(DsbA) is 3.1 μM, and V_{max} is 5.0 nmol of ubiquinone per nmol of DsbB/s.

absorption change between ubiquinone and its corresponding ubiquinol ($\Delta\epsilon_{275}$ = 12.25 mM^{-1}). Fig. 1 shows that DsbB efficiently reduces Q_0C_{10} as measured by the absorbance change at 275 nm. No reduction of ubiquinone is observed in the absence of DsbB. Only when DsbB is added to the reaction mixture in catalytic amounts did we observed reduction of ubiquinone. Enzymatic activities were derived from the initial slopes of absorbance decrease. For ubiquinone reduction we observed an activity of 278 nmol of ubiquinone per nmol of DsbB per min. This activity agrees well with the activity derived from the fluorescence assay measured under the same conditions (data not shown). Thus, this new assay can be employed to determine the activity of DsbB.

In order to characterize the ubiquinone reductase activity of DsbB, we measured initial velocities of ubiquinone reduction at various concentrations of Q_0C_{10}. The concentration of DsbA was held constant at 20 μM, whereas the concentration of the quinone was varied (Fig. 2*A*). To obtain kinetic constants the curves were fit to the Michaelis-Menten equation. When Q_0C_{10} was used as a substrate the apparent K_m was 2.0 μM, whereas V_{max} was 5.0 nmol of DsbA per nmol of DsbB/s. The concentration of DsbA was at 20 μM (0.42 mg/ml), and a less than 15% increase in velocity was observed at 40 μM reduced DsbA (data not shown). This suggests that at a concentration of 20 μM the reduced DsbA is close to saturating conditions. To verify this further, we measured the K_m for DsbA under saturating concentrations of Q_0C_{10}. The K_m value for DsbA was previously reported to be 10–13 μM (11, 28). This value was derived from

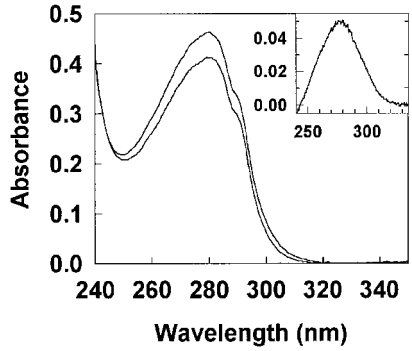

FIG. 3. **UV spectra of DsbB suggest that DsbB contains bound ubiquinone.** The UV spectrum of DsbB was recorded from 240 to 340 nm in 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside (*upper trace*). Solid sodium borohydride was added to the cuvette, and the spectrum of the reduced protein (*lower trace*) was recorded after 5 min of incubation at room temperature. The *inset* shows the difference spectra of oxidized minus reduced DsbB.

measurements of DsbB activity in membranes. An apparent K_m value for the highly purified system was determined to be 3.1 μM (Fig. 2*B*). This value was measured under saturating concentrations of quinone (25 μM) and in the presence of 0.1% dodecyl maltoside. The apparent k_{cat}/K_m values for ubiquinone is $3{\cdot}10^6$ M^{-1} s^{-1}, making quinones very specific substrates for DsbB. To our knowledge, DsbB has an undescribed enzymatic activity; it catalyzes the formation of a disulfide bond by the reduction of ubiquinone.

DsbB Contains Bound Coenzyme Q-8 (Ubiquinone-40) after Purification—The observation that DsbB catalyzes the reduction of quinones led us to analyze the quinone-binding properties of DsbB. We first decided to test if DsbB contains a quinone bound after purification. Oxidized quinones can be detected by a change in absorption at 275 nm upon reduction by addition of sodium borohydride. Ubiquinones show an absorbance peak at 275 nm, which decreases upon reduction of the quinone to the quinol. Fig. 3 shows the UV spectra of purified DsbB before and after the addition of sodium borohydride ($NaBH_4$). We observed an 11% decrease in the absorption at 275 nm after $NaBH_4$ was added to the cuvette. We attributed this change in absorbance to the reduction of a ubiquinone bound to DsbB. The addition of the $NaBH_4$ did not change the absorbance of the buffer blanked against water or DsbA, which served as a control (data not shown). From this absorbance change and given that the absorbance coefficient of coenzyme Q-8 is 12.25 mM^{-1} we calculated that the purified DsbB contains 0.5 molar eq of bound quinone.

In order to obtain additional proof for binding of ubiquinone to DsbB, we extracted DsbB with hexane, which should denature the protein and extract any bound quinones. The extract was analyzed by HPLC. The comparison of the elution profile of the DsbB extract (Fig. 4*a*) with the elution profiles from known quinone standards (*c* and *d*) indicates that the sample extracted from DsbB contains coenzyme Q-8. Mixtures of the DsbB extract and purified coenzyme Q-8 migrate as a single peak (Fig. 4*b*). The identification of coenzyme Q-8 as the DsbB-bound quinone agrees very well with the fact that this quinone is the predominant quinone species present in *E. coli* membranes under aerobic growth conditions (25). From the peak area we calculated a molar ratio of 0.6 mol of coenzyme Q-8 (ubiquinone Q_0C_{40}) bound per mol of DsbB, consistent with the value derived from the difference spectra shown in Fig. 1.

We wanted to test to see how many high affinity quinone-binding sites were present in DsbB. Quinone-binding sites in proteins can be titrated by addition of external quinone, with the binding being followed by an absorbance change of the

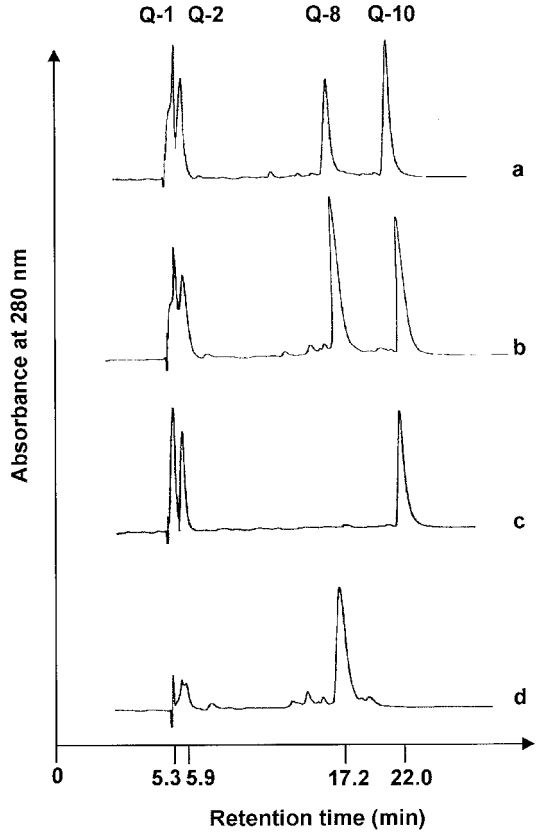

FIG. 4. **Identification that the quinone bound to DsbB is ubiquinone Q-8 by HPLC analysis.** DsbB was extracted with organic solvents as described under "Experimental Procedures." The DsbB extract was dissolved in ethanol, mixed with various purified quinones, and loaded onto a reverse phase C8-HPLC column. Coenzymes Q-1, Q-2, Q-8, and Q-10 served as standards. Profile *a* consists of Q-1, Q-2, DsbB extract, and Q-10. Profile *b* consists of Q-1, Q-2, DsbB extract + added Q-8, and Q-10. Profile *c* consists of Q-1, Q-2, and Q-10. Profile *d* is only purified Q-8. The DsbB extract was judged to contain Q-8 based on the observations that the retention time for the DsbB-extracted sample is the same as for the Q-8 standard (*a* and *d*) and because a single, enhanced peak at the Q-8 position results when a mixture of the DsbB extract and Q-8 was applied (*b*). From the peak area of the known Q-8 standard a molar ratio of bound quinone to DsbB of 0.6 was calculated.

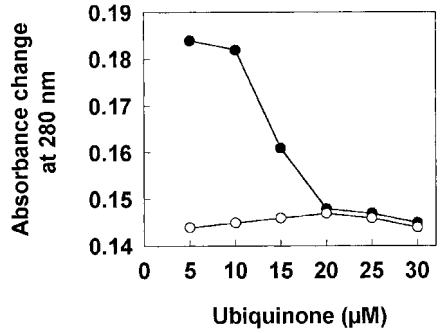

FIG. 5. **Titration of quinone-binding site of DsbB shows that DsbB has a single high affinity quinone-binding site.** DsbB was washed extensively with 50 mM sodium phosphate, 1% sodium cholate. After this procedure the amount of bound ubiquinone was 0.3 mol (not shown). The titration was performed in the same buffer by adding $Q_0C_{10}Br$ in 1-μl increments from a 5 mM stock solution. After each addition, the sample was incubated for 5 min, and the change in absorbance at 280 nm was recorded. A change in absorption greater than that shown for the buffer control indicates the burial of ubiquinone in a protein environment. This change is specific for ubiquinone-binding proteins (35). The cuvette containing DsbB at 22 μM (*solid circles*) shows a greater change in absorbance than the buffer controls (*open circles*) after quinone additions of up to 20 μM $Q_0C_{10}Br$. The DsbB contained 0.3 mol of quinone per mol of DsbB (7 μM of quinone bound) prior to any additions. We conclude that ~27 μM quinone is bound at saturation to 22 μM DsbB, a ~1:1 ratio.

TABLE I
DsbB specifically reoxidizes DsbA

No DsbB activity was detected with drRNase, GSH, and reduced DsbC as substrates at DsbB concentrations up to 250 nM. We determined the lower limit of detection of DsbB activity as 0.5 nM DsbB under standard assay conditions. Thus, reduced DsbA is oxidized at a rate at least 500-fold faster than the other three substrates tested.

Potential DsbB substrate	Concentration	Ubiquinone	Activity
	μM		
drRNase	10	20	<0.5
GSH	100	20	<0.5
DsbC, reduced	10	20	<0.5
DsbA, reduced	10	20	243

quinone upon binding to the protein (35). Fig. 5 demonstrates that the presence of DsbB strongly affects the spectral properties of externally added $Q_0C_{10}Br$. $Q_0C_{10}Br$ has been shown to be a better ubiquinone analogue for the study of protein-quinone-interaction (36). The different spectral properties of $Q_0C_{10}Br$ in the presence of DsbB indicate the transfer of $Q_0C_{10}Br$ from an aqueous to a more hydrophobic environment. We attribute this absorbance change to the binding of ubiquinone to DsbB. The absorbance change of $Q_0C_{10}Br$ upon interaction with DsbB reaches a saturation level (*closed circles*). After addition of 20 μM, $Q_0C_{10}Br$ showed the spectral properties that are typically observed for ubiquinone in the aqueous buffer control (*open circles*). The DsbB concentration was 22 μM. By taking into account that the protein contained 0.3 mol of bound ubiquinone after the detergent exchange, the titration with external quinone provides evidence that the quinone site of DsbB can be titrated to a 1:1 molar ratio. This demonstrates that DsbB possesses a single, highly specific quinone-binding site.

Reconstitution of the Entire Oxidative Protein Folding Pathway in Vitro—DsbA is a rather nonspecific, but powerful, oxidant capable of acting on many folding proteins, including eukaryotic proteins expressed in *E. coli*. DsbB presumably has a much more limited substrate specificity. It functions to reoxidize DsbA *in vitro* and *in vivo*. The severe disulfide defect present in strains that lack DsbA is good evidence that DsbB is incapable of directly oxidizing folding proteins *in vivo* (5). Our ability to measure directly DsbB activity by its ubiquinone reductase activity now allows us to test directly the rate at which DsbB can oxidize unfolded proteins and small molecule thiol-containing compounds. As a potential substrate for DsbB we chose ribonuclease A, which contains four disulfide bonds in its native state. The oxidative refolding of RNase has been well studied making this protein an excellent model substrate for our *in vitro* system (29). We tested if catalytic quantities of DsbB (0.1 μM) were capable of quinone reduction after the addition of 10 μM denatured, reduced RNase A. We were, however, not able to detect any DsbB-catalyzed quinone reduction in the absence of DsbA (Table I). Only after addition of a catalytic amount of oxidized DsbA (0.1 μM), a decrease in absorbance at 275 nm was observed (Fig. 6). This provides good biochemical evidence that DsbB is not capable of directly oxidizing RNase A under conditions where DsbA can function. This is further evidence that DsbA acts as the direct donor of disulfides during protein refolding. Reduced DsbA generated from the oxidation of RNase serves as the substrate of DsbB, which in turn reduces quinone. The reduction of quinones was measured as a decrease in absorbance at 275 nm. We followed

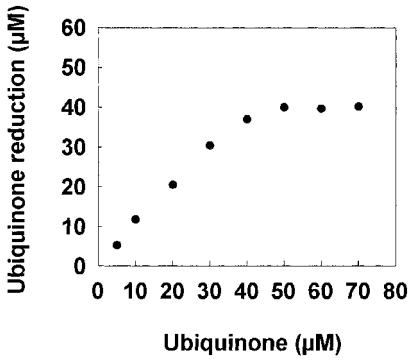

FIG. 6. **One ubiquinone is reduced per disulfide bond formed by DsbB.** The assay buffer contained 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 0.1% *n*-dodecyl maltoside. Oxidized DsbA was present at a concentration of 1 μM, and DsbB was at 0.1 μM. Note that both DsbA and DsbB are present in catalytic amounts in respect to ribonuclease A, which served as a model substrate protein. Ribonuclease A was denatured and reduced as described under "Experimental Procedures." Ribonuclease A was added to a final concentration of 10 μM, which corresponds to 80 μM of free thiols. The reduction of ubiquinone was followed at 275 nm until completion in order to determine the total amount of ubiquinone used up by the DsbA-DsbB system. Up to a concentration of 40 μM ubiquinone, the amount of ubiquinone that becomes reduced is directly proportional to the ubiquinone concentration. Above 40 μM, no additional reduction of ubiquinone is observed suggesting that 40 μM ubiquinone and the presence of the DsbA-DsbB system are sufficient to oxidize completely 40 μM of disulfides in ribonuclease A.

this absorbance change until the reaction was complete in order to determine the stoichiometry of the reaction (Fig. 6). The total amount of reduced ubiquinone was determined and plotted against the initial concentration of ubiquinone. The titration curve shows that the reduction of 40 μM quinones is necessary to oxidize completely 10 μM RNase A. Since RNase A contains 4 disulfides, it appears that one quinone gets reduced for every disulfide bond formed. That ribonuclease was completely oxidized was verified by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate trapping and non-reducing SDS-polyacrylamide gel electrophoresis (data not shown).

DsbA is regarded by some workers (20, 30), including the authors of this paper, as a powerful but rather nonspecific oxidant that is not necessarily capable of directing correct disulfide formation. Others (21) have shown that in the presence of glutathione redox buffers DsbA acts as a disulfide oxidoreductase, capable of refolding denatured and reduced proteins *in vitro*. Although widely used in DsbA-catalyzed folding reactions, glutathione is a non-physiological reoxidant. Because of its periplasmic location, DsbA is unlikely to encounter glutathione or any other small molecule disulfides, unless they are present in the media. Our *in vitro* system may more closely mimic the *in vivo* situation since DsbA is reoxidized by its physiological reoxidant, DsbB, which in turn is reoxidized by its *in vivo* reoxidant, ubiquinone. This *in vitro* reconstitution of the disulfide catalytic machinery present *in vivo* provides a suitable platform to test if DsbA is capable of correctly oxidizing a protein that contains multiple disulfides. To determine if the ribonuclease reoxidized by our *in vitro* disulfide catalytic system had been properly refolded, we simply determined how much RNase activity was regained. Native RNase catalyzes the hydrolysis of cCMP that can be measured by an increase in absorbance at 296 nm. After ribonuclease has been incubated with the DsbA-DsbB system, RNase activity was determined in the presence of cCMP. Fig. 7 shows that ribonuclease A, which has been reoxidized by the DsbB-DsbA system, shows only low activity indicating that the bulk of the ribonuclease had not been refolded properly, presumably due to misoxidation of thiols yielding nonnative disulfide bonds.

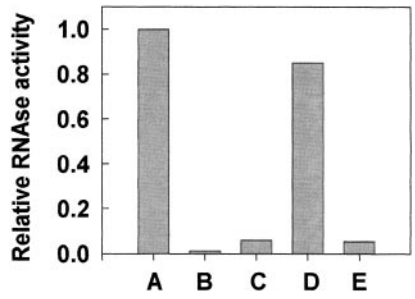

FIG. 7. **Reactivation of ribonuclease A depends on the presence of the disulfide isomerase DsbC.** The reactivation of ribonuclease was followed by measuring its cCMP hydrolyzing activity at 296 nm. The fraction of refolded and correctly oxidized ribonuclease was determined by comparing activities to the native protein. Each sample was incubated for 2 h at 25 °C in 50 mM sodium phosphate, 300 mM NaCl, 0.1% *n*-dodecyl maltoside followed by 1:10 dilution into the same buffer containing 5 mM cCMP. *A,* 10 μM native ribonuclease; *B,* 10 μM drRNase, 0.1 μM DsbA, 50 μM Q_0C_{10}; *C,* 10 μM drRNase, 0.1 μM DsbA, 50 μM Q_0C_{10}, 0.1 μM DsbB; *D,* 10 μM drRNase, 0.1 μM DsbA, 50 μM Q_0C_{10}, 0.1 μM DsbB, 10 μM reduced DsbC; *E,* 10 μM drRNase, 0.1 μM DsbA, 50 μM Q_0C_{10}, 0.1 μM DsbB, 10 μM oxidized DsbC. This experiment was performed three times; a typical result is shown.

This shows that DsbA does not have sufficient isomerase activity to oxidize proteins properly. DsbC shows significant disulfide isomerase activity and is thought be the prokaryotic equivalent of eukaryotic protein disulfide isomerase. To test if DsbC could isomerize this misoxidized RNase to generate active RNase, we added DsbC to the reaction. When 10 μM reduced DsbC is added to the DsbA-DsbB system, the reactivation yield of RNase increases to 90% suggesting that ribonuclease which had been oxidized by DsbA contained nonnative disulfide bonds (Fig. 7). DsbC was added in a reduced form so it could attack wrongly formed disulfides. We did not observe an increase in the reactivation of ribonuclease, when oxidized DsbC was added to the reactivation mixture. This clearly demonstrates that DsbC functions as a disulfide isomerase and excludes the possibility that the chaperone activity of DsbC is responsible for the observed RNase reactivation. We recently showed that the DsbC homologue DsbG is a chaperone and that the chaperone activity is independent of its redox state (37).

It is an interesting question of how the cell keeps its oxidative DsbA-DsbB pathway kinetically isolated from the isomerization/reduction DsbC-DsbD pathway. DsbA is mainly in the oxidized form *in vivo*, whereas DsbC is in the reduced form, suggesting that the cell has managed to avoid the futile cycle of DsbA oxidizing DsbC. DsbC is only very slowly oxidized by DsbA *in vitro*, suggesting that it is kinetically isolated (31). We also predict that DsbB should not be capable of rapidly oxidizing DsbC. We reasoned that DsbC is not oxidized by DsbB directly, because DsbC would not be capable of catalyzing disulfide rearrangement in our *in vitro* system.

In order to obtain direct evidence that DsbB does not oxidize DsbC, we incubated 10 μM reduced DsbC with 0.1 μM DsbB in the presence of quinone. Table I shows that DsbB does not reoxidize reduced DsbC in a quinone-dependent manner. This shows that the DsbC-dependent isomerization pathway is separated from the oxidative DsbA-DsbB pathway.

DsbB does not seem to catalyze the quinone-dependent oxidation of either ribonuclease A or reduced DsbC. To obtain further evidence that DsbB acts specifically on DsbA we chose the tripeptide GSH as a potential substrate for DsbB. As in the case with ribonuclease and DsbC, no quinone-dependent oxidation of GSH was observed at a DsbB concentration of 0.25 μM. As the detection limit of DsbB activity in our quinone reduction assay is about 0.5 nM, all three substrates tested,

drRNase, reduced DsbC, and GSH, are reoxidized at least 500-fold more slowly by DsbB than is DsbA.

DISCUSSION

Disulfide bonds are formed during the folding of many periplasmic proteins (1). By reconstituting the DsbA-DsbB system *in vitro*, we were able to identify the electron acceptor of DsbB to be quinones (6). In *E. coli*, quinones play a central part in the electron transport chain linking primary dehydrogenases to terminal enzyme complexes, which in turn catalyze the transfer of electrons onto final acceptors such as oxygen, nitrate, or fumarate (25). We showed that highly purified cytochrome *bd* or *bo* oxidase, which catalyze the final step of electron transport onto oxygen, were able to reoxidize DsbB *in vitro* (6). We wondered how DsbB could interact with two different substrates, cytochrome *bd* and *bo* oxidases. These oxidases, despite their similar name, are actually rather different proteins sharing no homology but sharing a quinone as a common substrate. We reasoned that it is more economical to postulate a single quinone-binding site on DsbB than two distinct binding sites, one for cytochrome *bd* oxidase and one for cytochrome *bo* oxidase. This was supported by our observation that it is possible to replace either of the oxidases by adding stoichiometric amounts of Q_0C_{10} or menadione to the assay system (6). Here we show that purified DsbB contains 0.6 mol of bound quinone per mol of protein. Since the DsbB purification protocol involves multiple columns and extensive dialysis, this implies that the quinone is rather tightly bound. The species of quinone bound to DsbB was determined to be coenzyme Q-8, the most abundant quinone found in the *E. coli* inner membrane under aerobic conditions. The ubiquinone-binding site can be titrated with externally added quinone to yield a 1:1 ratio. This demonstrates that DsbB possesses a single, highly specific quinone-binding site. In biological systems most disulfide chemistry studied is the chemistry of disulfide exchange. Little information is available describing their ultimate source. In cell-free systems, one common source is metal-catalyzed formation of disulfides directly from oxygen. This is unlikely to be important *in vivo*, because of its slow rate and because free concentrations of metal *in vivo* are vanishingly small. DsbB possesses a novel catalytic activity, which to our knowledge has not yet been described. It catalyzes the oxidation of a disulfide using oxidized quinones as an electron donor. DsbB is apparently the major source of disulfides in prokaryotes; mutations in *dsbB* have severe defects in disulfide bond formation (5). The other Dsb proteins have important roles in disulfide exchange, but none seem to actually create disulfides *de novo*. We did not observe spontaneous reaction of DsbA and ubiquinone under our assay conditions. The reaction between thiols and quinones is rather complex as the redox reaction competes with arylation of the thiol groups. DsbB is capable of accelerating the redox reaction specifically and very efficiently. The apparent k_{cat}/K_m value for the DsbB-catalyzed reaction between quinone and reduced DsbA is $4 \cdot 10^6\ M^{-1}\ s^{-1}$.

The identification of all the components required for the reoxidation of DsbA led us to reconstitute the DsbA-DsbB-quinone system with ribonuclease A as protein substrate. RNase A contains four disulfide bonds in its native state and has served as a model substrate for eukaryotic protein disulfide isomerase (29). When present in catalytic amounts, DsbB did not catalyze the formation of disulfide bonds of denatured and reduced ribonuclease. Only in the presence of catalytic amounts of DsbA was reduction of quinones observed. This clarifies the direction of disulfide and electron flow during oxidative protein folding; DsbA is the immediate donor of disulfides, but DsbB does not act on folding proteins in our assay system. This agrees very well with the *in vivo* situation as *dsbA* null mutants show a severe defect in disulfide bond formation even if DsbB is overexpressed (5). We also show that, under our assay conditions, DsbB does not catalyze the oxidation of the small peptide GSH to its disulfide-linked dipeptide form GSSG. This seems to make DsbA a rather specific substrate for DsbB. It has, however, been reported that thioredoxin and eukaryotic protein disulfide isomerase can rescue a *dsbA* null mutation in a DsbB-dependent manner. It was also observed that membranes containing DsbB reoxidize thioredoxin with a similar K_m as for DsbA *in vitro* (28). We attribute this slight promiscuity of DsbB to the fact that thioredoxin and PDI, which contains four thioredoxin like domains, have similar structures as does DsbA (9, 32).

It has been suggested that the disulfide isomerase DsbC is not reoxidized by DsbB and only slowly by DsbA (16, 31). Employing our highly purified system, we could show that the reoxidation of DsbC by DsbB is at least 500-fold slower than the reoxidation of DsbA. This is an important finding because DsbB, by discriminating DsbC from DsbA, makes possible the separation of the oxidative pathway from the isomerization pathway. How does DsbB distinguish between DsbA and DsbC? The recently solved crystal structure of DsbC revealed that dimeric DsbC contains two thioredoxin domains, which are joined via a dimerization domain (33). The active sites point toward the interior of a cleft formed by the dimer. We speculate that the active sites of DsbC are not accessible for DsbB and are therefore resistant to oxidation by DsbB.

The DsbA-DsbB system was able to oxidize denatured, reduced ribonuclease. There was, however, only little catalytic activity of ribonuclease detectable after oxidation by the DsbA-DsbB system. For ribonuclease there are numerous possible disulfide intermediates, only one of which represents the native state of the protein. The fact that after addition of reduced DsbC catalytic activity of ribonuclease was regained emphasizes that it is not sufficient to provide oxidizing equivalents for disulfide bond formation with multiple disulfide bonds. Efficient protein folding with proteins containing multiple disulfide bonds requires an enzyme that shuffles incorrectly formed disulfide bonds toward the ones found in the native state of the protein. DsbC and the very similar DsbG carry out disulfide bond isomerization. The presence of DsbC increases the yield of heterologous expressed eukaryotic proteins containing multiple disulfide bonds (5). However, in *E. coli*, proteins with more than two disulfide bonds are rare making the need for efficient isomerization of disulfide bonds less urgent than in the eukaryotic endoplasmic reticulum (5).

In summary, our results further elucidate the mechanism of disulfide bond formation and how it is linked to the electron transport chain. By reducing ubiquinone, DsbB plays a central part in this pathway by creating disulfides from the reduction of quinone. It has been suggested that the C*XX*C motif of DsbB is reoxidized by the electron transport chain (34). In the light of our results, it is very likely that quinones directly reoxidize this pair of cysteines. Biochemical analysis is currently being performed in order to get a better understanding of which residues on DsbB interact specifically with quinones.

Acknowledgments—We thank members of the Bardwell Laboratory for helpful suggestions.

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